

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07H 21/00, A61K 31/70</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/29116</b> <b>(43) International Publication Date:</b> 14 August 1997 (14.08.97)
<b>(21) International Application Number:</b> PCT/GB97/00327 <b>(22) International Filing Date:</b> 6 February 1997 (06.02.97)  <b>(30) Priority Data:</b> 9602326.2                      6 February 1996 (06.02.96)                      GB  <b>(71) Applicant (for all designated States except US):</b> CRUACHEM LIMITED [GB/GB]; Todd Campus, West of Scotland Science Park, Acre Road, Glasgow G20 0UA (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> REESE, Colin, Bernard [GB/GB]; 21 Rozel Road, Clapham, London SW4 0EY (GB). RAO, Mothe, Vaman [IN/US]; 826 Charlestown Meadows Drive, Westborough, MA 01581 (US).  <b>(74) Agent:</b> MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		<b>(81) Designated States:</b> DE, ES, GB, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES  <b>(57) Abstract</b>  There is provided a process for the solid phase synthesis of phosphorothioate oligonucleotides in which a dimeric phosphoramidite synthon is used to extend the oligonucleotide chain, the synthon having an optionally protected thioester group in its internucleotide linkage. Novel dimeric phosphoramidite synthons having such a thioester group are also described. The process enables increased yield of the oligonucleotide of interest with enhanced separation from impurities. The presence of the thioester linkage stabilises the oligonucleotide end product, facilitating its use as an anti-sense oligonucleotide analogue for gene therapy.		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

1     **SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES**

2

3     The present invention relates to dinucleotide  
4     phosphoramidites having a non-bridging sulphur group  
5     attached to the phosphorus moiety, the synthesis of  
6     these compounds and their use in the synthesis of  
7     phosphorothioate oligonucleotides.

8

9     The standard methodology for oligonucleotide synthesis  
10    relies upon solid phase chemistry. In a typical  
11    synthetic protocol phosphoramidites are added in a  
12    stepwise manner to an initial immobilised nucleoside,  
13    with protecting and deprotecting steps as necessary in  
14    each cycle. The process is now automated and is  
15    normally able to produce  $10^{-6}$  mol quantities of the  
16    desired end product. A suitable methodology is  
17    described by Beaucage in Methods in Molecular Biology,  
18    Vol 20, Protocols for Oligonucleotides and Analogues,  
19    ed Agrawal, Humana Press, Totawa, 1993, pages 33-61.

20

21    More recently, the synthesis of S-alkyl esters of 2'-  
22    deoxyribonucleoside 3'-phosphorothioates has been  
23    reported (see Liu et al, J. Chem. Soc. Perkin Trans 1 :  
24    1685-1694 (1995)) and the use of such compounds in the  
25    synthesis of oligonucleotide phosphorothioates was

1 suggested.

2  
3 Phosphorothioate oligonucleotides are regarded as the  
4 first generation of antisense oligonucleotide analogues  
5 which have been successfully tested *in vitro* and *in*  
6 *vivo* as inhibitors of gene expression (see,  
7 "Oligonucleotides: Antisense Inhibitors of Gene  
8 Expression", Ed. Cohen, Macmillan, London, 1989 and  
9 "Prospects for Antisense Nucleic Acid Therapy of Cancer  
10 and AIDS", Ed. Wickstrom, Wiley-Liss, New York, 1992).  
11 At present, a few uniformly modified phosphorothioate  
12 oligonucleotides are in human clinical trials and have  
13 the potential to be used as approved drugs. (see,  
14 Ravikumar *et al*, Bioorganic & Medicinal Chemistry  
15 Lett.: 2017-2022 [1994]). Large quantities, multiple  
16 gram to multiple kilogram, of high purity  
17 phosphorothioate oligonucleotides are required at low  
18 and acceptable cost suitable for therapeutic  
19 applications.

20  
21 Phosphorothioate oligonucleotides are isoelectronic  
22 analogues of natural oligonucleotides in which one of  
23 the non-bridging internucleotide oxygen atoms is  
24 replaced by a sulphur atom. The solid phase synthesis  
25 of phosphorothiate oligonucleotides has been achieved  
26 using H-phosphonate chemistry (see, Froehler *et al*,  
27 Tetrahedron Lett. 5575-5578 [1986]) where only one  
28 sulphur transfer step is required after assembling the  
29 desired sequence to convert all the internucleotide  
30 linkages to phosphorothioates, or the phosphoramidite  
31 approach (see, Stec *et al*, J. Am. Chem. Soc., 6077-6079  
32 [1984] and Rao *et al*, Tetrahedron Lett., 6741-6744  
33 [1994]) where monomeric phosphoramidites are added in  
34 each synthetic cycle and a stepwise sulphurisation  
35 instead of iodine oxidation step in an otherwise  
36 standard synthetic cycle is used to assemble the

1 desired phosphorothioate oligonucleotides. The solid  
2 phase monomeric phosphoramidite chemistry is routinely  
3 used to synthesize phosphorothioate oligonucleotides  
4 (on micromole to millimole scale) as considerable  
5 efforts have been expended in enhancing the efficiency  
6 of the synthesis such as (i) the use of improved  
7 synthetic cycle protocols and solid supports (see,  
8 Ravikumar *et al*, Bioorganic & Medical Chemistry Lett.,  
9 2017 [1994]) (ii) sulphur transfer reagents (see Rao *et al*,  
10 Tetrahedron Lett., 6741 (1994) and references cited  
11 therein), (iii) capping and deblocking reagents (see,  
12 Agrawal *et al*, Tetrahedron Lett., 8565 [1994]).  
13 However, problems still remain both in terms of  
14 consistent yields and quality of the final  
15 oligonucleotide phosphorothioate. In particular the n-  
16 1 and n+1 impurities are very similar to the full  
17 length product "n" and vary from batch to batch,  
18 especially when reduced excesses of monomeric  
19 nucleoside phosphoramidite synthons are used in each  
20 synthetic cycle. In order to meet the quality  
21 specifications of the full length phosphorothioate  
22 oligonucleotide needed for therapeutic applications,  
23 which are very high, it is necessary to repeatedly  
24 purify the product, free from n-1 and n+1 impurities.  
25 Consequently the process will result in lowering the  
26 yield of the full length product and hence the overall  
27 process might not be cost effective.

28  
29 Whilst the potential utility of phosphorothioates has  
30 been recognised there still remains a need for an  
31 effective and efficient manufacture of these complex  
32 molecules. In particular it has not previously been  
33 recognised that dimeric or larger phosphoramidite  
34 blockmers could be advantageously applied in their  
35 synthesis via solid phase chemistry.

36

1 In order to alleviate some of these problems, recent  
2 efforts have been focused on investigating the  
3 feasibility of the large scale synthesis of  
4 phosphorothioate oligonucleotides by the  
5 phosphotriester approach in solution (see Reese *et al*,  
6 J. Chem. Soc. Perkin Trans., 1685 [1995] and Imbach *et*  
7 *al*, Antisense Res. Dev. 39 [1995]. While this approach  
8 offers definite advantages over the solid phase  
9 monomeric phosphoramidite chemistry, in that:

10

11 (i) it is more suitable for scale-up for synthesis in  
12 much larger quantities, (e.g. millimoles to mole +  
13 scale)

14

15 (ii) it allows addition of two or more nucleotide  
16 residues at a time (i.e., block synthesis)

17

18 (iii) it offers the choice of purifying fully  
19 protected blockmers at different stages prior to  
20 assembling the desired sequence and

21

22 (iv) it allows much easier purification of the final  
23 product,

24

25 it requires further development.

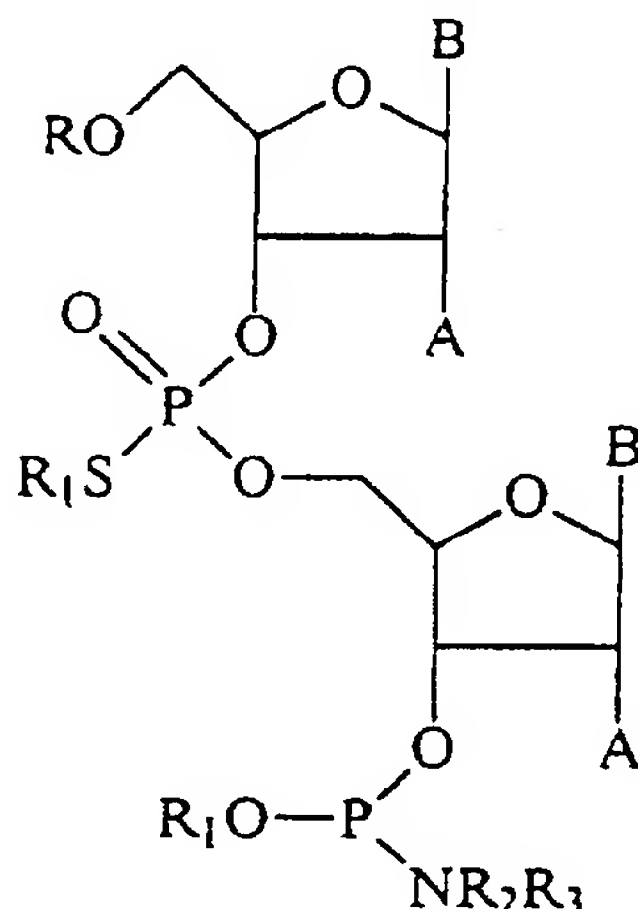
26

27 However, the solid phase phosphoramidite approach  
28 (useful for micromole to millimole scale synthesis) can  
29 be improved by the addition of a dimeric  
30 phosphoramidite synthon instead of a monomeric  
31 phosphoramidite synthon during the synthetic cycle and  
32 this forms the basis of the present invention. The  
33 dimeric phosphoramidite approach would achieve an  
34 increased yield (as the number of steps required to  
35 produce a particular oligonucleotide will be reduced)  
36 and enhanced separation of the desired oligonucleotide

from the impurities (as their use results in n-2 and n+2 impurities instead of n-1 and n+1 impurities) due to the greater difference in size.

The present invention provides an improved process for the solid phase synthesis of phosphorothioate oligonucleotides using dinucleotide phosphoramidite synthons containing the S-protected phosphorothioate ester internucleotide linkage and a 3'-phosphoramidite functional group.

The present invention provides novel compounds of formula I



wherein

B represents a heterocyclic amine base or a derivative thereof;

R represents an acid labile protecting group;

R<sub>1</sub> represents a protecting group, preferably selected from the group consisting of 2-cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl and 4-nitrophenyl;



1      $R_2$  represents a blocking or protecting group;

2

3      $R_3$  represents a blocking or protecting group; and

4

5     A represents a hydrogen atom, or an alkoxy, allyloxy or  
6     suitably protected hydroxy group.

7

8     The dinucleotide phosphoramidite of formula I can be  
9     used in conventional automated solid phase synthesis to  
10    produce phosphorothioate oligonucleotides.

11

12    Thus, the present invention also provides a process for  
13    producing an oligonucleotide having at least one  
14    phosphorothioate linkage, said process comprising  
15    providing a compound of formula I above for reaction  
16    with the terminal nucleoside of the nucleotide chain  
17    located at the solid phase to assemble the nucleotide  
18    chain. As used herein the term "nucleotide chain"  
19    includes a single nucleoside located at the solid phase  
20    which will itself be the terminal group available for  
21    reaction.

22

23    Group R is desirably 4,4'-dimethoxytrityl, but any  
24    other suitable protecting group may also be used.

25

26    Groups  $R_2$  and  $R_3$  may each independently be an alkyl or  
27    aryl group.

28

29    The heterocyclic base of group B may be, for example a  
30    purine, such as adenine, guanine or derivatives  
31    thereof, or a pyrimidine, such as cytosine, uracil,  
32    thymine or derivatives thereof. As derivatives may be  
33    mentioned alkylated derivatives (especially methylated  
34    derivatives) and halogenated derivatives, but are not  
35    specially limited thereto. Uracil and derivatives  
36    thereof may be especially convenient for use.



- 1 The present invention will now be further described
- 2 with reference to the following non-limiting Examples.

1     Example 1a

2  
3     Triethylammonium salt of 5'-O-(4,4'-  
4     dimethoxytrityl)thymidine S-(2-cyanoethyl)  
5     3'-phosphorothioate (see Reese et al, J. Chem. Soc.  
6     Perkin Trans. 1: 1685 [1995])  
7

8     To a stirred solution of 1,2,4-triazole (8.28g, 0.126  
9     mol) in anhydrous tetrahydrofuran (250ml) was added  
10    triethylamine (18.08ml, 0.13 mol) and phosphorus  
11    trichloride (3.5ml, 40 mmol) at approximately -35°C  
12    (methanol-CO<sub>2</sub> bath). The reaction was stirred for 15  
13    minutes, after which 5'-O-(4,4'-dimethoxytrityl)  
14    thymidine (5.546g, 10.2 mmol) in tetrahydrofuran  
15    (200ml) was added. After a further 30 minutes,  
16    triethylamine - water (60ml, 1:1 v/v) was added  
17    dropwise with stirring and the reaction mixture was  
18    allowed to warm up to ambient temperature. The solvent  
19    was removed under reduced pressure. The residue was  
20    dissolved in chloroform (500ml) and washed with 0.5M  
21    triethylammonium bicarbonate (2 x 250ml). The organic  
22    layer was dried (MgSO<sub>4</sub>) and evaporated. The residue was  
23    co-evaporated with acetonitrile (3 x 100ml), and then  
24    dissolved in anhydrous dichloromethane (180ml). N-(2-  
25    Cyanoethylthio)phthalimide (3.09g, 13.3 mmol) was  
26    added, followed by N-methylmorpholine (6.67ml, 60 mmol)  
27    and chlorotrimethylsilane (5.07ml, 40 mmol). The  
28    mixture was allowed to stir at ambient temperature.  
29    After 3 hours, the reaction mixture was poured into  
30    0.5M triethylammonium bicarbonate (200ml). The organic  
31    layer was separated and the aqueous layer was extracted  
32    with dichloromethane (200ml). The combined organic  
33    layers (dried over MgSO<sub>4</sub>) were evaporated. The residue  
34    was purified by short-column chromatography and the  
35    product-containing fractions, which were eluted with  
36    CHCl<sub>3</sub>-MeOH (90:10 to 85:15 v/v), were evaporated under

1 reduced pressure. The residue was dissolved in  
2 chloroform (40ml) and the title compound was obtained  
3 by precipitation from petroleum ether (b.p. 30-40°C,  
4 400ml) as a colourless solid (8.10g).  
5  
6  $\delta_H$  [ $CD_3$ ]<sub>2</sub>SO]: 1.18 (1.5 H, t, J = 7.3 Hz), 1.36 (3 H,  
7 s), 2.40 (2 H, m), 2.69 (2 H, m), 2.83 (2 H, m), 3.03  
8 (1 H, q, J = 7.2 Hz), 3.17 (1 H, m), 3.32 (1 H, m),  
9 3.74 (6 H, s), 4.19 (1 H, m), 4.91 (1 H, m), 6.23 (1 H,  
10 t, J = 7.2 Hz), 6.89 - 7.41 (13 H, m), 7.52  
11 (1 H, s) 11.40 (1 H, s).  
12  
13  $\delta_P$  [ $CD_3$ ]<sub>2</sub>SO]: 13.9 ppm  
14 HPLC data:  $R_1$  = 9.65 minute (Programme 1)  
15 Column : ODS 5 $\mu$  (5 x 250 mm)  
16 Eluting Conditions : Curve Select : linear gradient,  
17 time of programme = 10 minutes; flow : 1.5 ml/minute;  
18 Initial conditions: 0.1M triethylammonium acetate  
19 (TEAA) buffer : acetonitrile (7:3, v/v)  
20 Final conditions: 0.1M TEAA buffer : acetonitrile  
21 (2:8, v/v)

1     Example 1b

2

3     Triethylammonium salt of N-benzoyl-5'-O-  
4     (dimethoxytrityl)deoxycytidine S-(2-cyanoethyl) 3'-  
5     phosphorothioate

6

7     This compound was prepared on the same scale and in  
8     precisely the same way as the thymidine derivative  
9     described above. N-benzoyl-5'-O-(dimethoxytrityl)  
10    deoxycytidine (6.336g, 10 mmol) was converted into the  
11    title compound (8.84g) as a colourless solid.

12

13     $\delta_H$  [ $CD_3$ )<sub>2</sub>SO]: 1.19 (6 H, t, J = 7.3 Hz), 1.36 (3 H, s),  
14    2.32 (1 H, m), 2.68 (1 H, m), 2.85 (2 H, m), 3.06 (4 H,  
15    q, J = 7.3 Hz), 3.41 (2 H, m), 3.75 (6 H, m), 4.29 (1  
16    H, m), 4.85 (1 H, m), 6.18 (1 H, t, J = 6.3 Hz), 6.90 -  
17    8.00 (19 H, m), 8.18 (1 H, d, J = 7.5 Hz) 11.31 (1 H,  
18    s).

19

20     $\delta_P$  [ $CD_3$ )<sub>2</sub>SO]: 13.2 ppm

21    HPLC data:  $R_1$  = 11.25 minutes (Programme 1)

1     Example 1c

2  
3     5'-O-(Dimethoxytrityl) thymidin-3'-yl-N-  
4     benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl)  
5     phosphorothioate

6  
7     A solution of triethylammonium salt of 5'-O-  
8     (dimethoxytrityl)thymidine-S-(2-cyanoethyl)-3'-  
9     phosphorothioate (2.012g, 2.5 mmol) (from Example 1a),  
10    N-benzoyldeoxycytidine (1.035g, 3.125 mmol) and 3-  
11    nitro-1,2,4-triazole (0.998g, 8.75 mmol) in pyridine  
12    (25 ml) was concentrated to dryness under reduced  
13    pressure. This process was repeated twice more and the  
14    residue was dissolved in dry pyridine (20ml).  
15    Mesitylene-2-sulfonyl chloride (1.64g, 7.5 mmol) was  
16    added and the solution was allowed to stir for 30  
17    minutes. The reaction was quenched with saturated  
18    aqueous sodium bicarbonate (2.5ml), and the products  
19    were partitioned between chloroform (50ml) and  
20    saturated aqueous sodium bicarbonate (150ml). The  
21    organic layer was separated and the aqueous layer was  
22    extracted with chloroform (4 x 30ml). The combined  
23    organic layers were dried (MgSO<sub>4</sub>) and evaporated under  
24    reduced pressure. The residue was co-evaporated with  
25    toluene (2 x 20ml) and then purified by short-column  
26    chromatography. The appropriate fractions, eluted with  
27    CHCl<sub>3</sub>-MeOH (98:2 to 96.5-3.5 v/v) were combined and  
28    evaporated under reduced pressure. A solution of the  
29    residue in chloroform (10ml) was added dropwise to  
30    petroleum ether (b.p. 30-40°C, 200ml) to give the title  
31    compound as a precipitate (1.57g, 61.8%).

32  
33     $\delta_H$  [CD<sub>3</sub>)<sub>2</sub>SO]: 1.45 (3 H, s), 2.15 (1 H, m), 2.35 (1 H,  
34    m), 2.57 (2 H, m), 2.90 (2 H, m) 3.10 (2 H, m), 3.31 (2  
35    H, m), 3.73 (6 H, s), 4.07 (1 H, m), 4.23 (2 H, m),  
36    4.32

1 (2 H, m), 5.23 (1 H, m), 5.56 (1 H, , d, J = 4.3 Hz),  
2 6.16 (1 H, m), 6.25 (1 H, m),  
3 6.87 - 8.00 (20 H, m), 8.15 (1 H, m), 11.27 (1 H, s),  
4 11.41 (1 H, s).  
5  
6 On treatment with D<sub>2</sub>O signals at 11.27, 11.41, 5.56 ppm  
7 diminished in intensity.  
8  $\delta_{\text{H}}$  [CD<sub>3</sub>)<sub>2</sub>SO]; 27.7, 28.0 ppm  
9 HPLC data: R<sub>1</sub> = 12.12 minutes, 12.27 minutes (programme  
10 1)

1     Example 1d

2

3     N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidin-3'-yl  
4     thymidin-5'-yl S-(2-cyanoethyl) phosphorothioate

5

6     A solution of the triethylammonium salt of N-benzoyl-  
7     5'-O-(dimethoxytrityl)deoxycytidine S-(2-cyanoethyl)  
8     3'-phosphorothioate (4.42g, 5 mmol) (from Example 1b),  
9     thymidine (1.519g, 6.25 mmol) and 3-nitro-1,2,4-  
10    triazole (2.00g, 17.5 mmol) in dry pyridine (20ml) was  
11    concentrated to dryness under reduced pressure. This  
12    process was repeated twice more and the residue was  
13    dissolved in dry pyridine (50ml). Mesitylene-2-  
14    sulfonyl chloride (3.28g, 15.0 meal) was added and the  
15    solution was allowed to stir for 30 minutes. The  
16    reaction was quenched with saturated aqueous sodium  
17    bicarbonate (me) and the products were partitioned  
18    between chloroform (100ml) and 0.5M triethylammonium  
19    bicarbonate (200ml). The organic layer was separated  
20    and the aqueous layer was extracted with chloroform (3  
21    x 50ml). The combined organic layers were dried (MgSO<sub>4</sub>)  
22    and evaporated under reduced pressure. The residue was  
23    co-evaporated with toluene (3 x 20ml) and then purified  
24    by short-column chromatography. The appropriate  
25    fractions, eluted with CHCl<sub>3</sub>-MeOH (98:2 to 97:3 v/v)  
26    were combined and evaporated under pressure. A  
27    solution of the residue in chloroform (15ml) was added  
28    dropwise to petroleum ether (b.p. 30-40°C, 300ml) to  
29    give the title compound as a precipitate (3.06g, 60%).

30

31     $\delta_{\text{H}}$  [CD<sub>3</sub>)<sub>2</sub>SO]: 1.79 (3 H, s), 2.15 (2 H, m), 2.48 (1 H,  
32    m), 2.79 (2 H, m), 2.90 (2 H, m) 3.00 (2 H, m), 3.38 (2  
33    H, m), 3.74 (6 H, s), 3.99 (1 H, m), 4.34 (4 H, m),  
34    5.15  
35    (1 H, m), 5.52 (1 H, d, J = 4.5 Hz), 6.19 (2 H, m),  
36    6.89 - 8.03 (20 H, m), 8.18 (1 H, d, J = 7.4 Hz), 11.32



1 (1 H, z), 11.35 (1 H, s).

2

3 On treatment with D<sub>2</sub>O signals at 5.52, 11.32 and 11.35  
4 ppm diminished in intensity.

5  $\delta_p$  [CD<sub>3</sub>)<sub>2</sub>SO]; 27.7, 27.9 ppm

6 HPLC data: R<sub>1</sub> = 13.00 minutes, 13.13 minutes (Programme

7 1)

1     Example 2a

2

3     5'-O-(Dimethoxytrityl)-thymidin-3'-yl-3'-[(2-S-  
4     cyanoethyl)phosphoryl]-5'-N-benzoyl-2'-deoxycytidine-  
5     3'-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite

6

7     Abbreviation: T-P(s)-dC-CEPA

8

9     5'-O-(Dimethoxytrityl)thymidin-3'-yl N-  
10    benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl)  
11    phosphorothioate (8.20g, 8.151 mmol, 1 mol eq) (from  
12    Example 1c) was dissolved in dry dichloromethane (AR  
13    grade) (120ml) under an argon blanket, and allowed to  
14    stir for 5 minutes. To this solution was added  
15    diisopropyl-ammonium tetrazolide (1.394g, 1 mol eq)  
16    followed by bis-(N,N-diisopropylamino)-(2-O-cyanoethyl)  
17    phosphoramidite (4.914g, 2 mol eq) and the reaction  
18    mixture allowed to stir under an argon blanket for 1.5  
19    hours. The reaction was then washed with water (75ml),  
20    saturated NaCl solution (75ml) and saturated NaHCO<sub>3</sub>  
21    (75ml). The organic layers were separated and the  
22    aqueous layers were back extracted with dichloromethane  
23    (25ml) and the extract was added to the organic layers,  
24    which were then dried over anhydrous sodium sulphate  
25    (50g), filtered and then evaporated to a foam. The  
26    foam was then dissolved in dichloromethane (20ml) and  
27    purified on a silica chromatography column with a  
28    silica/product ration of 10:1. The column was first  
29    packed with 1% pyridine in dichloromethane, then once  
30    the product had been loaded onto the column it was  
31    eluted with dichloromethane (100ml), MeCN (2000ml), and  
32    10% MeOH in dichloromethane (250ml) to strip the  
33    column. The appropriate fractions were combined and  
34    evaporated under reduced pressure to a foam. The  
35    product was then dissolved in dichloromethane (50ml)  
36    and added dropwise to pentane (500ml) to give a

1 precipitate. This was then dissolved in  
2 dichloromethane and filtered through a 1 micron filter  
3 system, then evaporated to a foam and placed onto a  
4 freeze drier for a minimum of 8 hours. Yield = 7.5g,  
5 79.3%.  $\delta_p$  [CDCl<sub>3</sub>]: 26.85, 148.91, 149.52 ppm.  
6  
7 Analytical data from the compound formed is presented  
8 in Fig 1.

1     Example 2b

2

3     5'-O-(Dimethoxytrityl)-N-benzoyl-2'-deoxycytidine-3'-  
4     yl-3'-[2-S-cyanoethyl) phosphoryl]-5'-thymidine-3'-[2-  
5     cyanoethyl)-N,N-diisopropyl] phosphoramidite

6

7     Abbreviation: dC-P(S)-T-CEPA

8

9     5'-O-(Dimethoxytrityl)-N-benzoyl-deoxycytidin-3'-yl  
10    thymidin-5'-yl S-(2-cyanoethyl) phosphorothioate  
11    (8.00g, 7.952 mmol, 1 mol eq) (from Example 1d) was  
12    dissolved in dry dichloromethane (AR grade) (120ml)  
13    under an argon blanket and allowed to stir for 5  
14    minutes. To this solution was added  
15    diisopropylammonium tetrazolide (1.36g, 1 mol eq)  
16    followed by bis(N,N-diisopropyl-amino)-(2-O-cyanoethyl)  
17    phosphoramidite (4.794g, 2 mol eq), and the reaction  
18    mixture was allowed to stir under an argon blanket for  
19    1.5 hours. The reaction was then washed with water  
20    (75ml), saturated NaCl solution (75ml). The organic  
21    layers were separated and the aqueous layers were back  
22    extracted with dichloromethane (25ml) and the extract  
23    was added to the organic layers, which were then dried  
24    over anhydrous sodium sulphate (50g), filtered, and  
25    then evaporated to a foam. The foam was then dissolved  
26    in dichloromethane (20ml) and purified on a silica  
27    chromatography column with a silica/product ratio of  
28    10:1. The column was first packed with 1% pyridine in  
29    dichloromethane, then once the product had been loaded  
30    onto the column it was eluted with dichloromethane  
31    (100ml), MeCN (1000ml), and 10% MeOH in dichloromethane  
32    (250ml) to strip the column. The appropriate fractions  
33    were combined and evaporated under reduced pressure to  
34    a foam. The product was then dissolved in  
35    dichloromethane (50ml) and added dropwise to pentane  
36    (500ml) to give a precipitate. This was then dissolved

1 in dichloromethane and filtered through a 1 micron  
2 filter system, then evaporated to a foam and placed  
3 onto a freeze drier for a minimum of 8 hours. Yield =  
4 7.00g 73.0%.  $\delta_p$  [CDCl<sub>3</sub>]: 26.83, 149.09, 149.23 ppm

1     Example 3

2

3     Automated solid-phase synthesis of phosphorothioate  
4     oligonucleotides

5

6     Synthesis of phosphorothioate oligonucleotides were  
7     carried out using a Cruachem PS250 DNA/RNA synthesizer.  
8     Cruachem standard DNA phosphoramidites and reagents  
9     were used unless otherwise stated. One  $\mu$ m  
10    phosphorothioate synthetic cycle protocol in  
11    conjunction with a solution of 0.05M Beaucage reagent  
12    [3H-1,2-benzodithiol-3-one-1,1-dioxide] with 60 seconds  
13    reaction time for thiolation was used.

14

15    To evaluate the potential use of the present invention  
16    for the synthesis of phosphorothioate oligonucleotides,  
17    stringent coupling reaction conditions on the use of  
18    phosphoramidite synthons (3-4 excess molar equivalents)  
19    in conjunction with controlled pore glass containing a  
20    higher nucleoside loading (100  $\mu$ m/gram) were used. The  
21    compounds formed in Examples (2a) and (2b) were used as  
22    the corresponding solutions in anhydrous  $\text{CH}_3\text{CN}$  (0.1M).

23

24    To demonstrate the improvements of the present  
25    invention, a few phosphorothioate oligonucleotides were  
26    synthesized using the monomeric phosphoramidite  
27    synthons and the aforesaid conditions. Identical  
28    phosphorothioate oligonucleotide sequences were  
29    synthesized using the dimeric phosphoramidite synthons  
30    and after appropriate deprotection steps, the resulting  
31    oligonucleotides were compared.

1     Oligonucleotide sequences:

2

3     Seq 1D Nos 1 & 4       :     (TC)<sub>10</sub>T       -     21 mer

4     Seq 1D Nos 2 & 5       :     (CT)<sub>10</sub>T       -     21 mer

5     Seq 1D Nos 3 & 6       :     TCC TTC TCT CCT CTC TTC CTA   -  
6                               21 mer

7

8     Synthesis of Seq 1D Nos 1-3

9

10    The Sequences were produced using monomeric  
11    phosphoramidite synthons. The synthesis protocol  
12    therefore required 20 synthesis cycles and 20  
13    sulphurisation steps.

14

15    \*ACE   =   > 98%

16    (based on DMT cation assay)

17

18    Synthesis of Seq 1D Nos 4-6

19    The Sequences were produced using the dimeric  
20    phosphoramidite synthons (T-P(s)-dc-CEPA and  
21    dc-p(s)-T-CEPA). The synthesis protocol therefore  
22    required 10 synthesis cycles and 10 sulphurisation  
23    steps.

24

25    \*ACE   =   > 98%

26    (based on DMT cation assay)

27

28    \* *Average coupling efficiency*

29

30    Deprotection of Oligonucleotide Sequences:

31    (a) Seq 1D Nos 1 to 3 synthesized using monomeric  
32       phosphoramidite synthons were released from the  
33       solid support and deprotected by treating with  
34       concentrated aqueous ammonia (1.0mL) at 55°C for  
35       12 hours. The ammoniacal solution was evaporated  
36       to a pellet under reduced pressure and the  
37       unpurified (crude) oligonucleotides were analysed.



1        oligonucleotide with anhydrous pyridine (1.0 mL)  
2        using vacuum centrifugation. Once dried, the  
3        material was treated with a solution of DBU (1,8-  
4        Diazabicyclo[5, 4,0]-undec-7-ene) in anhydrous  
5        pyridine (5:95, v/v 1.0ml) for 2 hours at 30°C.  
6        The solvents were then removed and the residue was  
7        then treated with concentrated aqueous ammonia  
8        (1.0ml) at 55°C for 12 hours. The ammoniacal  
9        solution was evaporated to a pellet under reduced  
10       pressure and the unpurified (crude)  
11       oligonucleotides were analysed.

12  
13       HPLC (Ion Exchange) analysis:

14  
15       Ion-exchange HPLC analysis of phosphorothioate  
16       oligodeoxy-nucleotides was carried out using a Gilson  
17       712 Gradient system with dual pumps and fitted with a  
18       Gilson 117 UV Detector (280nm). A 5 micron Nucleopac  
19       PA100 column (5 x 250 mm) was used with eluents [A] :  
20       20 mM Tris-HCl buffer, pH = 8.0 and [B] : 400 mM sodium  
21       perchlorate in buffer [A].

22  
23       The results are shown in Figs 2 to 4.

24  
25       Fig 2 shows a comparison of anion-exchange (NucleoPac  
26       PA-100) chromatograms of unpurified 5'-O-DMT-on  
27       phosphorothioate oligomers (TC)<sub>10</sub>T 21-mer (Seq 1D Nos 1  
28       and 4). Fig 2A gives the results for the 21-mer  
29       synthesised with monomeric phosphoramidites (Seq 1D No  
30       1) which has a product purity of 68.5%. Fig 2B gives  
31       the results for the 21-mer synthesised with dimeric  
32       phosphoramidites (Seq 1D No 4) which has an increased  
33       product purity of 78.0%.

34  
35       Fig 3 shows a comparison of anion-exchange (NucleoPac  
36       PA-100) chromatograms of unpurified 5'-O-DMT-on

1 product purity of 78.0%.

2

3 Fig 3 shows a comparison of anion-exchange (NucleoPac  
4 PA-100) chromatograms of unpurified 5'-O-DMT-on  
5 phosphorothioate oligomers (CT)<sub>10</sub>A 21-mer (Seq 1D Nos 2  
6 and 5). Fig 3A gives the results for the 21-mer  
7 synthesised with monomeric phosphoramidites Seq 1D No  
8 2) which have a product purity of 74.0%. Fig 3B gives  
9 the results for the 21-mer synthesised with dimeric  
10 phosphoramidites (Seq 1D No 5) which has an increased  
11 product purity of 83.0%.

12

13 Fig 4 shows a comparison of anion-exchange (NucleoPac  
14 PA-100) chromatograms of unpurified 5'-O-DMT-on  
15 phosphorothioate oligomers (TCC TTC TCT CCT CTC TTC  
16 CTA) 21-mer (Seq 1D Nos 3 and 6). Fig 4A gives the  
17 results for the 21-mer synthesised with monomeric  
18 phosphoramidites (Seq 1D No 3) which have a product  
19 purity of 73.8%. Fig 4B gives the results for the 21-  
20 mer synthesised with dimeric phosphoramidites (Seq 1D  
21 No 6) which has an increased product purity of 85.5%.

22

23 Fig 5 is a comparison of <sup>31</sup>P NMR spectra of unpurified  
24 5'-O-DMT-on phosphorothioate oligomers for Seq 1D Nos 3  
25 and 6.

26

27 A: synthesised using monomeric phosphoramidites (Seq  
28 1D No 3)

29 B: synthesised using S-dimeric phosphoramidites (Seq  
30 1D No 6).

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

(A) NAME: CRUACHEM LTD

(B) STREET: WEST OF SCOTLAND SCIENCE PARK, TODD CAMPUS,  
ACRE ROAD

(C) CITY: GLASGOW

(E) COUNTRY: UK

(F) POSTAL CODE (ZIP): G20 0UA

## (ii) TITLE OF INVENTION: COMPOUNDS

## (iii) NUMBER OF SEQUENCES: 6

## (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

## (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: GB 9602326.2

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTCTCTCTC TCTCTCTCTC T

21

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

24

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTCTCTCTCT CTCTCTCTCT A

21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCTTCTCTC CTCTCTTCCT A

21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/label= PHOSPHOROTHIOAT

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTCTCTCTC TCTCTCTCTC T

21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified\_base

(B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)

(D) OTHER INFORMATION: /mod\_base= OTHER  
/label= PHOSPHOROTHIOAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTCTCTCTCT CTCTCTCTCT A

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified\_base

(B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)

(D) OTHER INFORMATION: /mod\_base= OTHER  
/label= PHOSPHOROTHIOAT

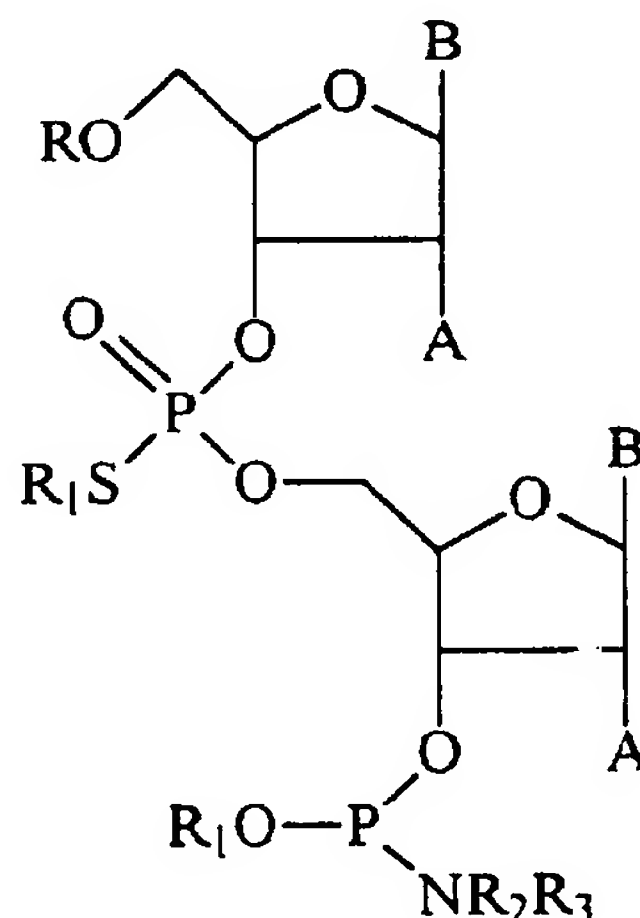
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TCCTTCTCTC CTCTCTTCCT A

21

## 1 CLAIMS

- 2
- 3 1. A process for the solid phase synthesis of
- 4 phosphorothioate oligonucleotides, said process
- 5 comprising the addition of at least one dimeric
- 6 phosphoramidite synthon during the synthetic
- 7 cycle, wherein said dimeric phosphoramidite
- 8 synthon comprises in its internucleotide linkage
- 9 an optionally protected thioester group.
- 10
- 11 2. A process as claimed in Claim 1 wherein said
- 12 dimeric phosphoramidite synthons are used as
- 13 reactants in each synthetic cycle.
- 14
- 15 3. A process as claimed in either one of Claims 1 and
- 16 2 wherein said thioester group present in said
- 17 internucleotide linkage is protected by a 2-
- 18 cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or
- 19 4-nitrophenyl group.
- 20
- 21 4. A dimeric phosphoramidite synthon being a compound
- 22 of Formula I:
- 23
- 24
- 25
- 26
- 27
- 28
- 29
- 30
- 31
- 32
- 33
- 34
- 35
- 36

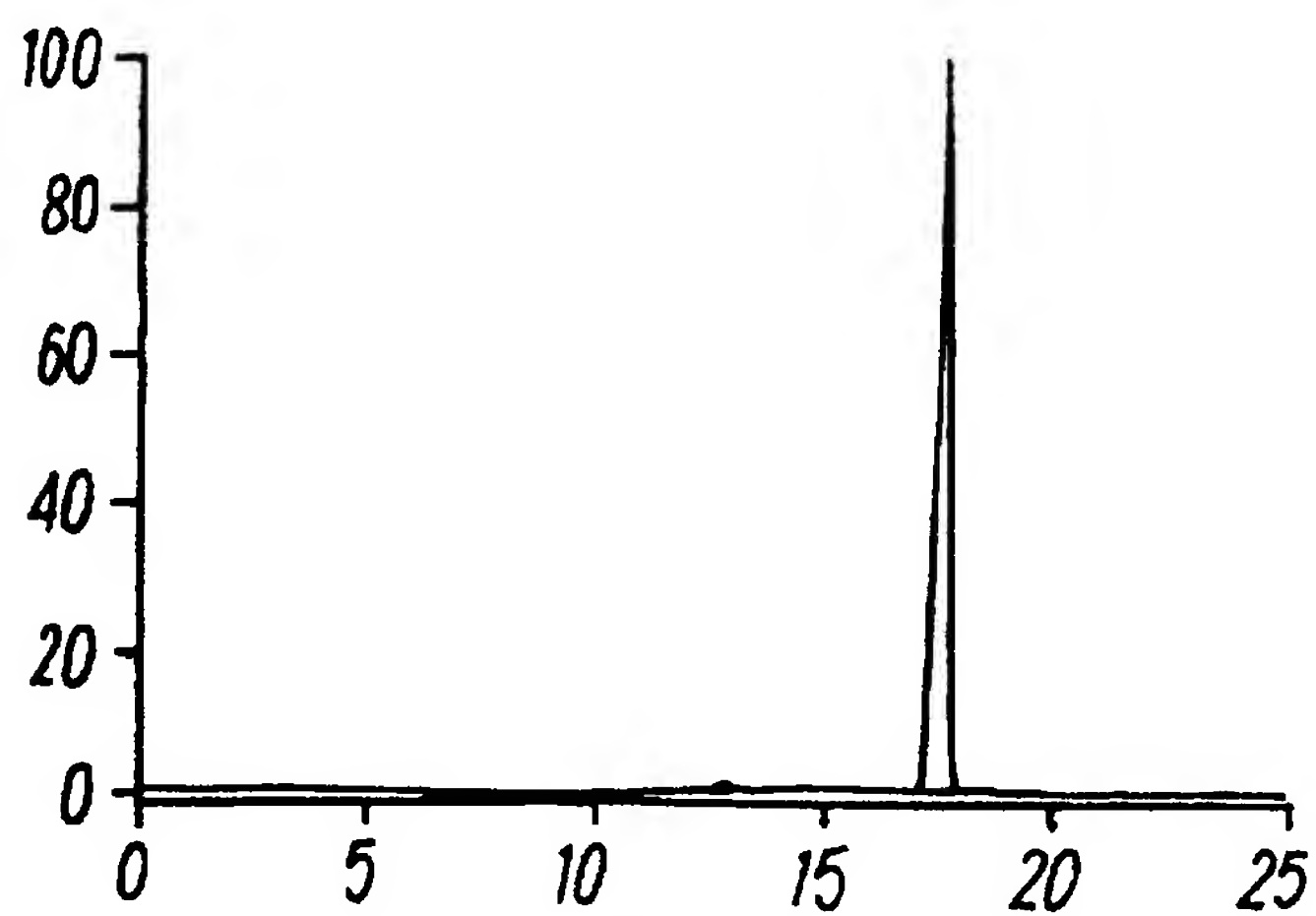
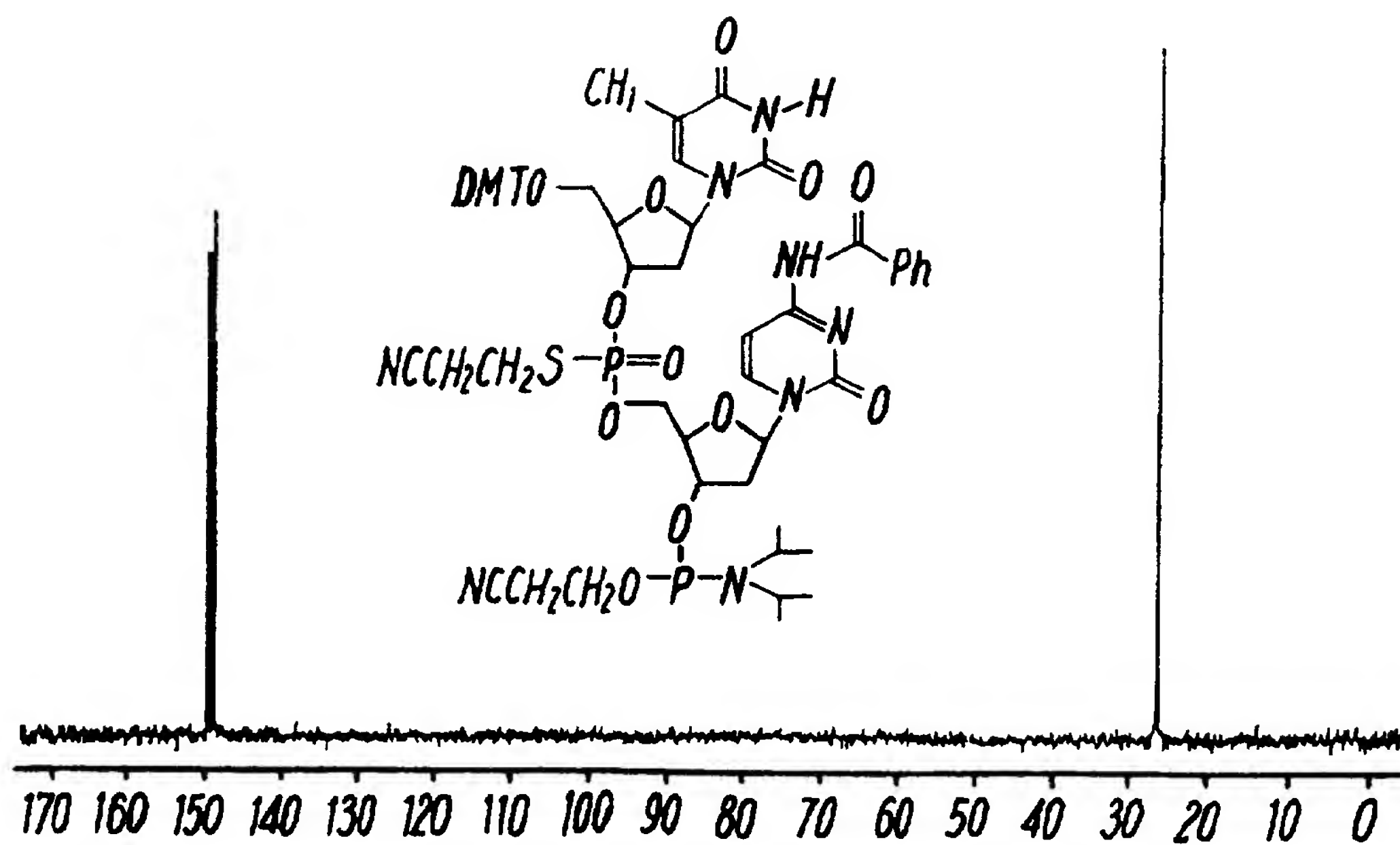


- 1           wherein  
2           B represents a heterocyclic amine base or a  
3           derivative thereof;  
4           R represents an acid labile protecting group;  
5           R<sub>1</sub> represents a protecting group;  
6           R<sub>2</sub> represents a blocking or protecting group;  
7           R<sub>3</sub> represents a hydrogen atom, or an alkoxy,  
8           allyloxy or suitably protected hydroxy group.  
9
- 10       5.    A compound as claimed in Claim 4 wherein group B  
11           is an adenine, guanine, cytosine, uracil or  
12           thymine base or the alkylated or halogenated  
13           derivatives of any of those bases.  
14
- 15       6.    A compound as claimed in Claim 5 wherein at least  
16           one group B is uracil or methylated uracil.  
17
- 18       7.    A compound as claimed in any one of Claims 4 to 6  
19           wherein group R is a 4,4'-dimethoxytrityl group.  
20
- 21       8.    A compound as claimed in any one of Claims 4 to 7  
22           wherein each group R<sub>1</sub> is independently a 2-  
23           cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or  
24           4-nitrophenyl group.  
25
- 26       9.    A compound as claimed in any one of Claims 4 to 8  
27           wherein each group R<sub>2</sub> and group R<sub>3</sub> is independently  
28           an alkyl or aryl group.  
29
- 30       10.   Use of a compound as claimed in any one of Claims  
31           4 to 9 in the synthesis of phosphorothioate  
32           oligonucleotides.  
33
- 34       11.   Use of phosphorothioate oligonucleotides produced  
35           in accordance with the process of Claims 1 to 3 as  
36           anti-sense nucleotides for inhibition of gene

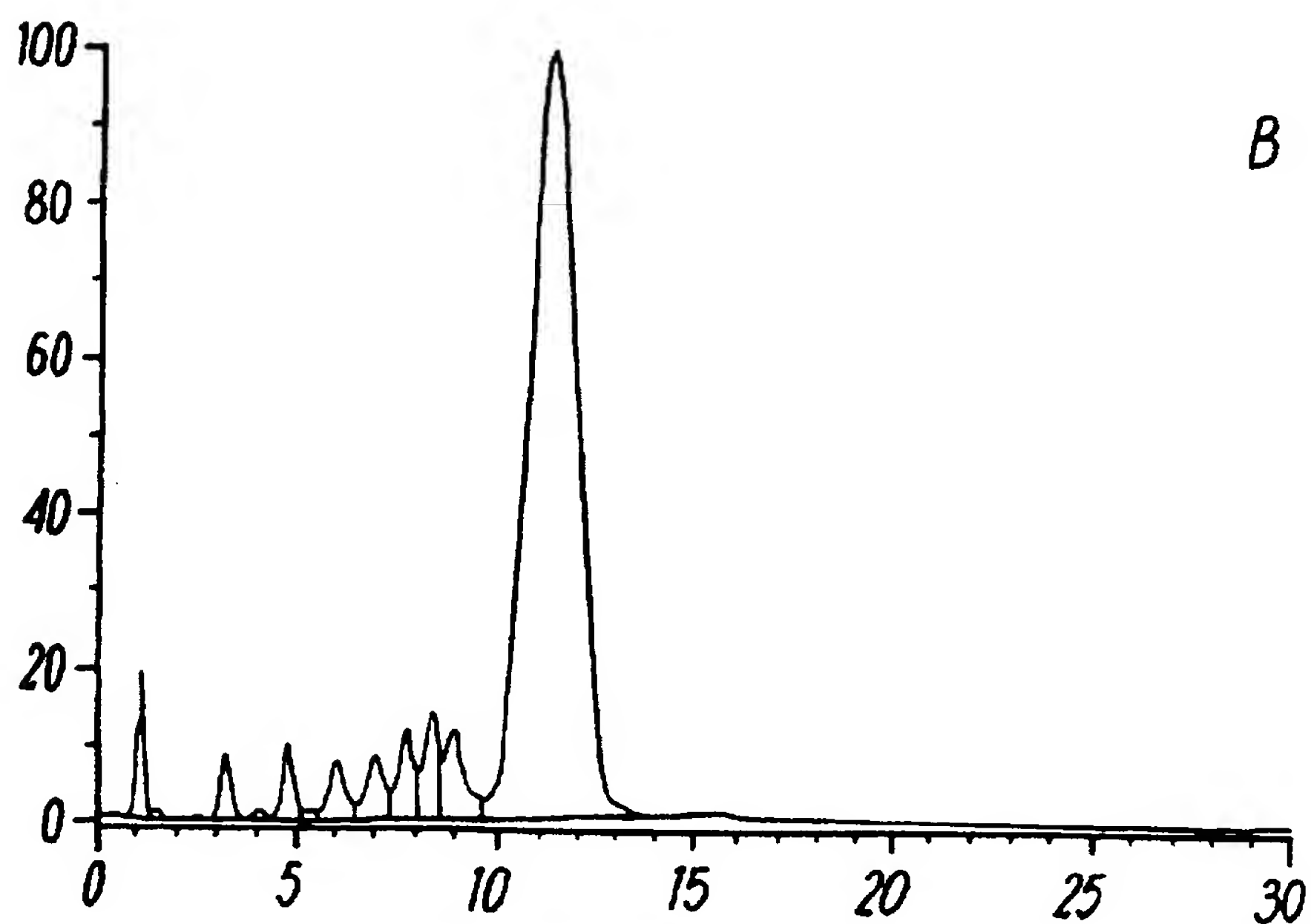
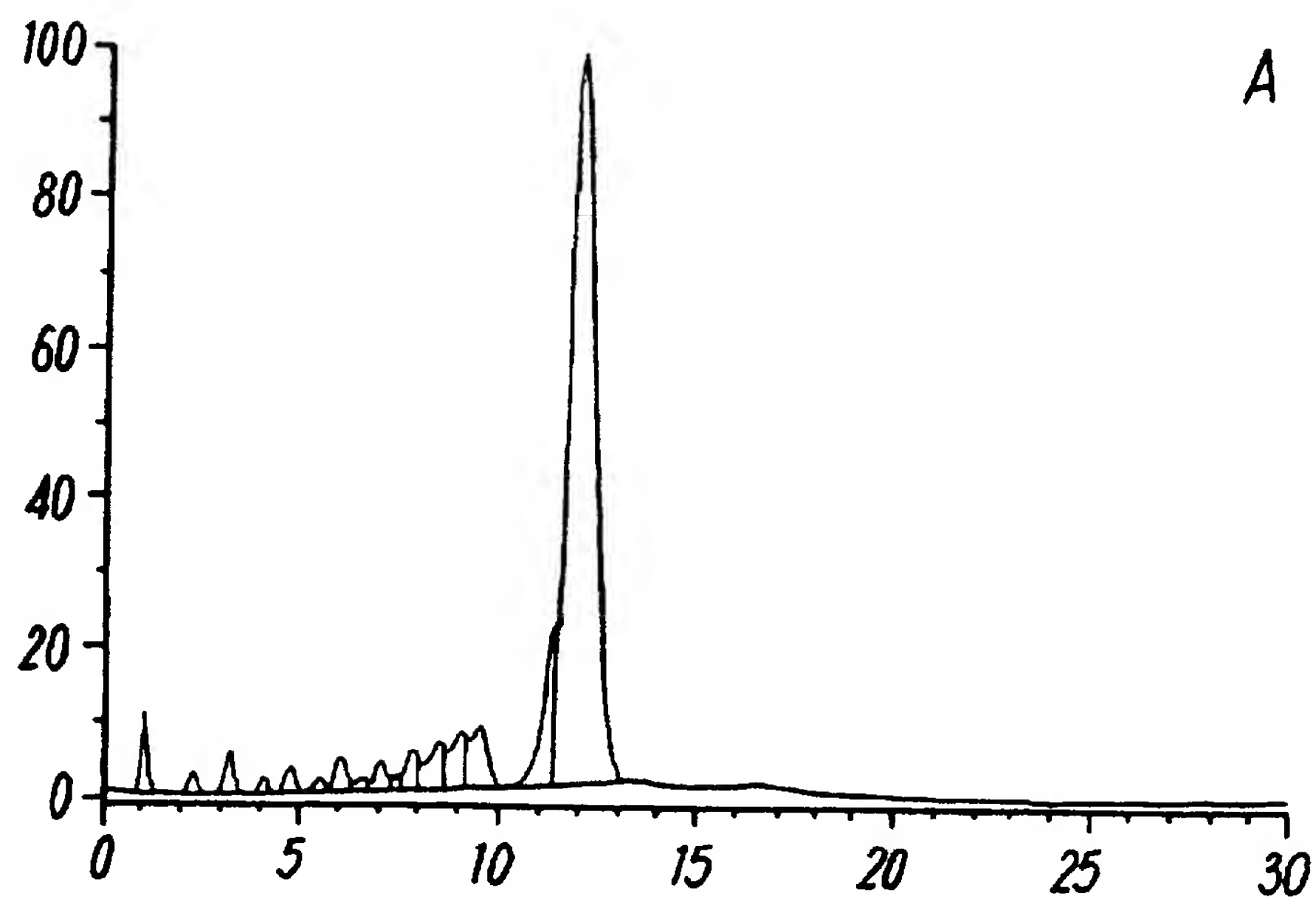


1 expression.

1 / 5

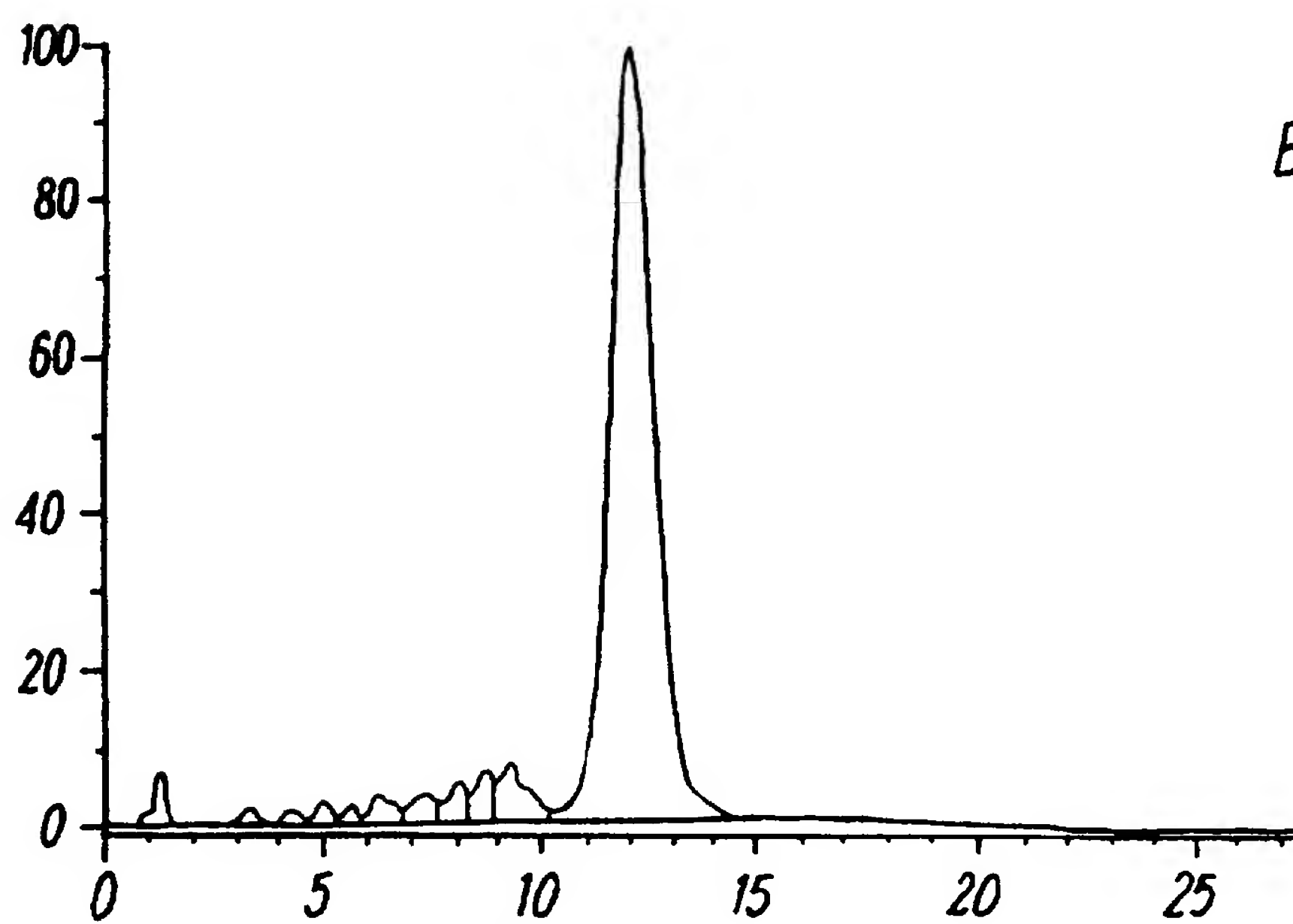
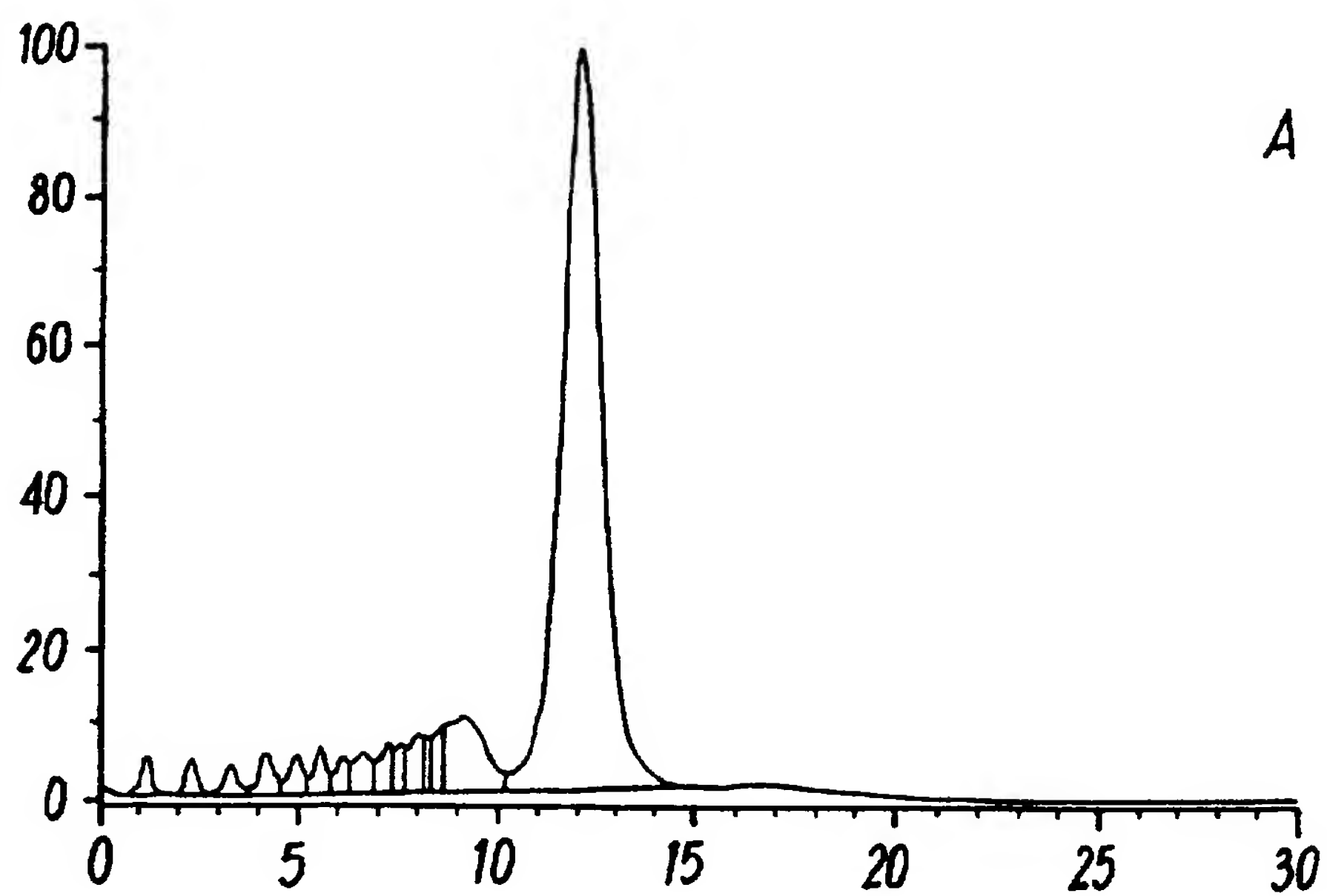
**FIG. 1**

2 / 5

**FIG. 2**

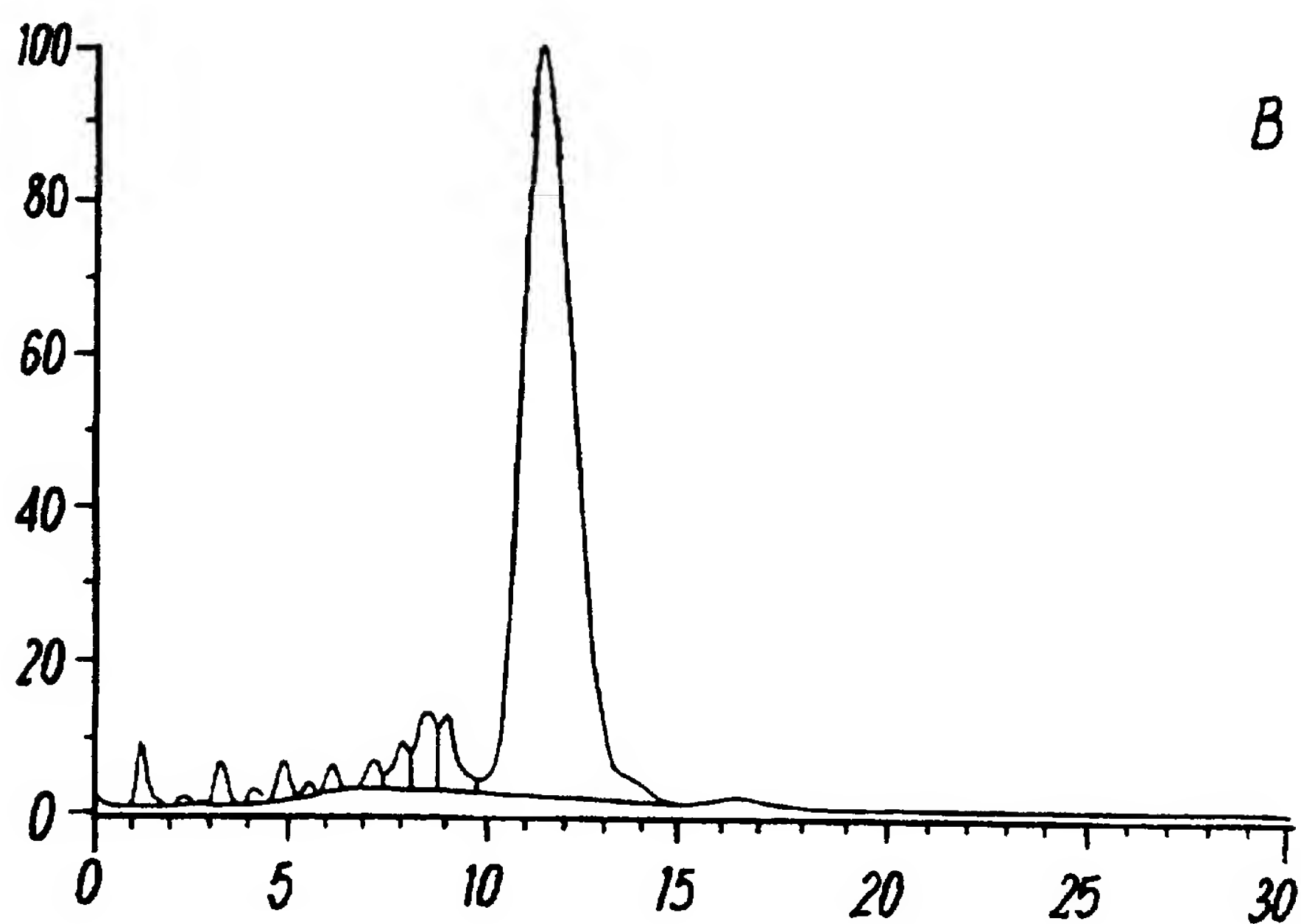
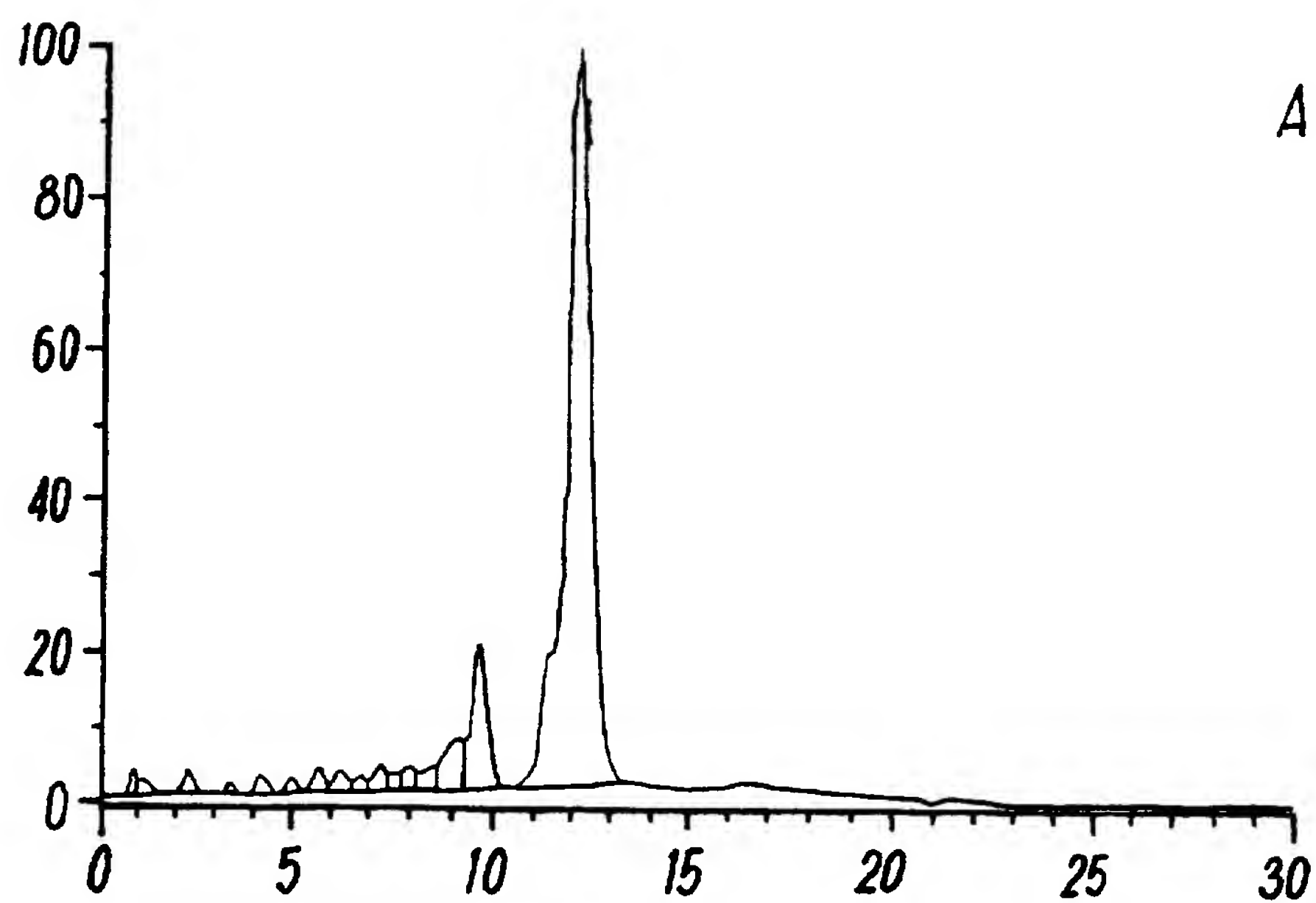
SUBSTITUTE SHEET (RULE 26)

3 / 5

**FIG. 3**

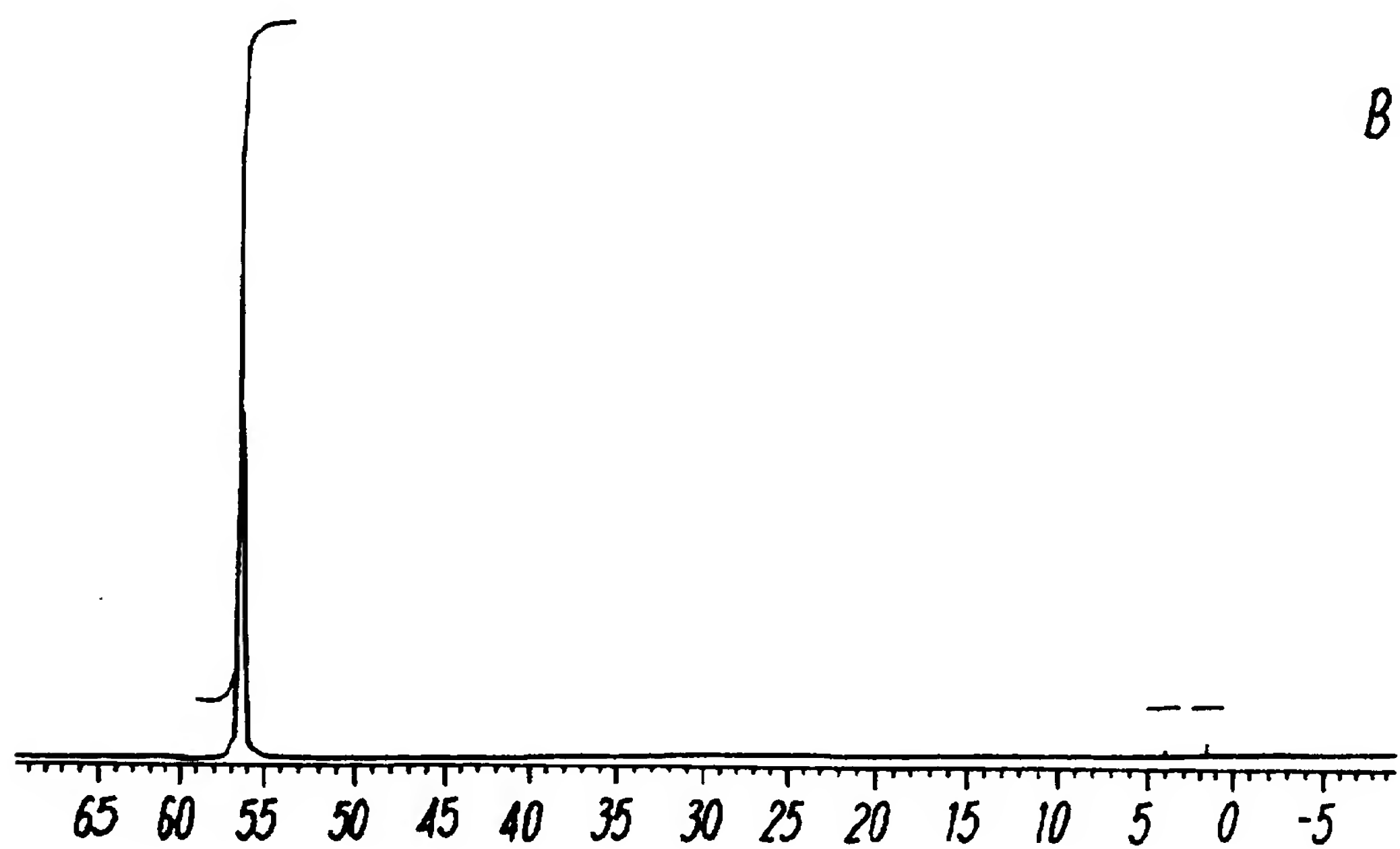
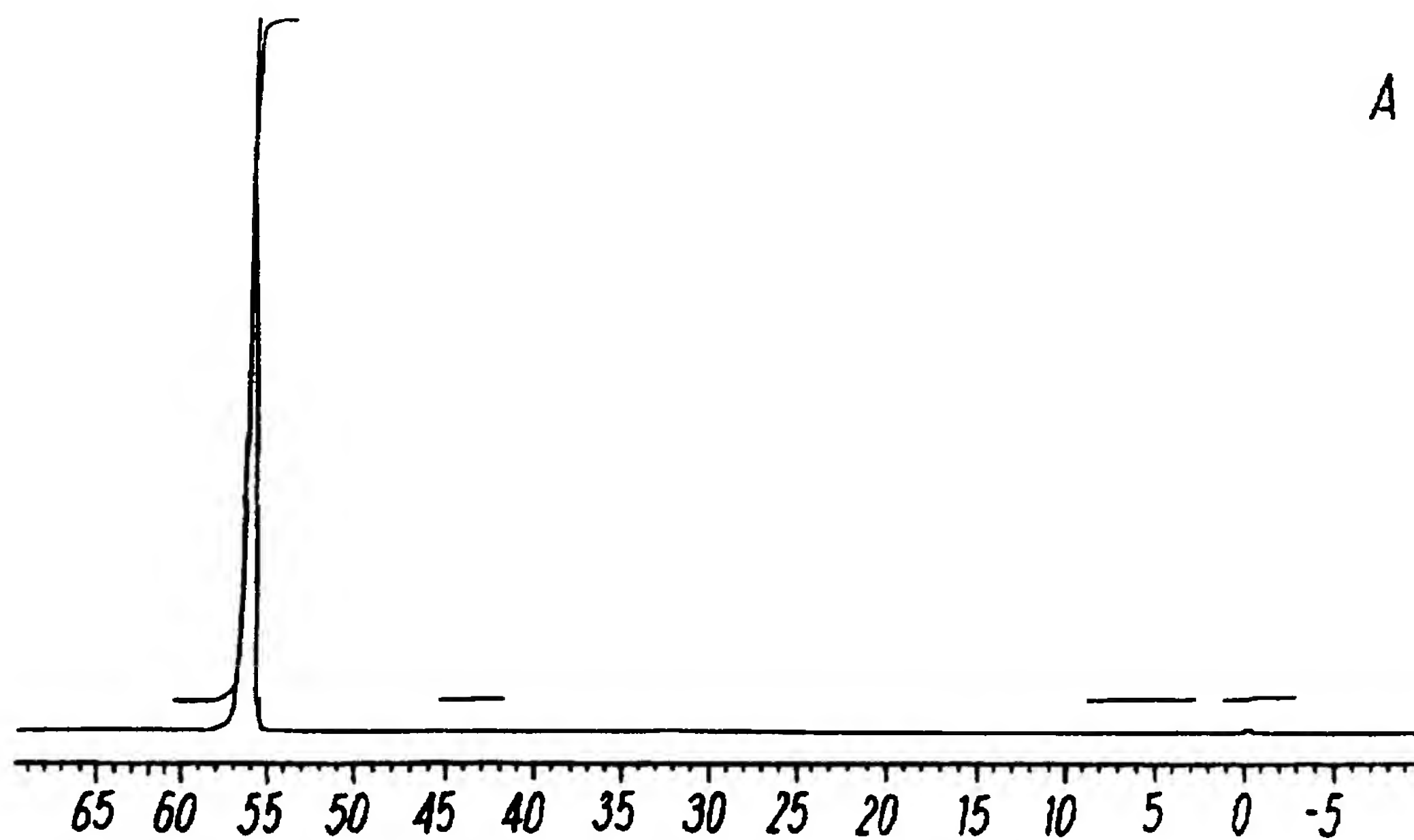
SUBSTITUTE SHEET (RULE 26)

4 / 5

**FIG. 4**

SUBSTITUTE SHEET (RULE 26)

5 / 5



**Fig. 5**

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

Internat Application No  
PC1/GB 97/00327A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEOSIDES & NUCLEOTIDES, vol. 11, no. 9, 1 January 1992, pages 1621-1638, XP000564715 ZBIGNIEW J LESNIKOWSKI: "THE FIRST STEREOCONTROLLED SYNTHESIS OF THIOOLIGORIBONUCLEOTIDE: (RPRP)- AND (SPSP)-UPSUPSU" see the whole document ---	1-11
A	WO 95 32980 A (ISIS PHARMACEUTICALS INC ;RAVIKUMAR VASULINGA (US); COLE DOUGLAS L) 7 December 1995 see the whole document ---	1,4
A	WO 95 14029 A (BECKMAN INSTRUMENTS INC) 26 May 1995 see the whole document ---	1,4
-/-		



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

12 June 1997

Date of mailing of the international search report

24-06-1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Moreno, C



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/00327

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 668 777 A (CARUTHERS MARVIN H ET AL) 26 May 1987 see the whole document ---	1,4
A	US 5 151 510 A (STEC WOJCIECH J ET AL) 29 September 1992 see the whole document -----	1,4

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 1/GB 97/00327

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9532980 A	07-12-95	US 5571902 A AU 2657095 A EP 0766688 A	05-11-96 21-12-95 09-04-97
WO 9514029 A	26-05-95	US 5616700 A	01-04-97
US 4668777 A	26-05-87	US 4415732 A US 4973679 A AU 551324 B AU 8199782 A CA 1203237 A EP 0061746 A JP 1638762 C JP 57176998 A JP 63028439 B JP 1515844 C JP 63065677 B JP 63179889 A	15-11-83 27-11-90 24-04-86 30-09-82 15-04-86 06-10-82 31-01-92 30-10-82 08-06-88 24-08-89 16-12-88 23-07-88
US 5151510 A	29-09-92	EP 0524942 A WO 9116331 A US 5292875 A	03-02-93 31-10-91 08-03-94